CHROMOGENESIS IN CULTURES OF SPOROTRICHA*

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(WITH PLATES 2 AND 3)

The pigmentation of fungi has long attracted attention for various reasons. Many are highly colored and therefore readily attract the eye and excite the interest of the observer. To the systematist, color has furnished an easy means of classification, and biologic nomenclature is laden with color words. It is generally admitted however that a color basis is very unsatisfactory for purposes of classifying or naming organisms. As Buller[1] states, it would be interesting if some law of progressive coloration could be discovered; but no attempt to work out the phylogenesis of the color of spores has yet been made. Buller thinks that colorlessness is the primitive condition of spores and that pigments are only gradually developed, probably by a series of mutations.

As to the significance of color in fungi, little can be said. Pigments in certain plants may be protective, especially against light; but certainly many pigments are not thus protective, for they occur in places never exposed to light. Such pigments probably have no significance or purpose, and represent merely normal products of metabolic processes.

Since at present the subject of pigmentation of lower organisms is not well known, especially from the chemical standpoint, and further work along this line is needed, I wish to record some data on pigmentation which have been accumulating for several years in connection with certain studies that I have been making on a number of strains of the pathogenic fungus sporothrix.

In a paper[2] published in 1898, the pigmentation of a sporotrichum was noted by Schenck, who was the first to describe this organism and call attention to its pathogenicity for man and animals. In describing the growth on agar, he states: "In cultures of ten days and older, the growth is very thick; the surface is rough, corrugated, and stained a dark brown color, the shade at the periphery being deeper than in the center. The medium also is colored." Again,

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1. Researches on Fungi, 1909, p. 13.
2. Bull. Johns Hopkins Hosp., 1898, 9, p. 286.

in discussing the growth on sugar media, he states: "In cultures older than fourteen days, however, the growth continues longer and becomes heavier on glucose than on plain agar, and in all the sugar media there is more discoloration both of the growth and of the substratum." On potato, he notes that the edges of the growth become "discolored and the potato darkened." Dr. Erwin F. Smith, of the U. S. Department of Agriculture, Washington, who discussed the classification of the organism in Schenck's paper, also refers to the color, especially with respect to its significance in the differentiation of fungi.

Hektoen and Perkins,[3] who reported the second case of sporotrichosis on record, refer to the pigmentation of cultures as follows: "About the seventh day the growth, which has increased somewhat in thickness, becomes light-brownish in color, the margins being smooth and wavy, marked by shallow transverse grooves. Still later the growth becomes distinctly, and even dark brown, the surface wrinkled and velvety, in some cases covered by a dark fuzz. The medium becomes slightly brownish."

Later reports, especially by the French workers De Beurmann and Gougerot, have noted pigmentation of the sporothrix organisms found in that country. In Gougerot's excellent article,[4] he states that the colonies are at first whitish, with later a gradual discoloration appearing: "Milk-coffee like, chocolate-brown, or black-brown." He further states that the pigment is constant but varies in rapidity of formation.

Page, Frothingham, and Paige,[5] in this country, who were first to identify the disease in horses, noted that the strains of sporothrix obtained from this animal likewise produced abundant dark brown or black pigment. Excellent photographs illustrating this point accompany their article.

The strains obtained from rats by Lutz and Splendore in Brazil, those from mules observed by Carougeau in Madagascar, and that obtained from a dog in Paris by Gougerot and Caraven — all were pigment producers.

After a careful search in the reports on this disease, the statement may be made that all the strains of this organism thus far isolated whether from man or animals, when grown under proper conditions on suitable artificial media, produce a more or less distinct brown or blackish pigment.

Beyond noting its mere presence, investigators have done little or no work thus far on the nature of the pigment or on the conditions under which it is formed. It was thought wise therefore to inquire somewhat minutely into these questions.

The observations were made on eight different strains, obtained originally from both human and animal lesions. Sporotricha from France were kindly given me by Sabouraud and by Gougerot of Paris. From Dr. Kren of Vienna, I obtained a strain isolated from a human case that developed in Austria. K. F. Meyer kindly gave me an organism isolated from a horse suffering from the infection. The other strains were isolated from human cases of sporotrichosis in the United States.

3. Jour. of Exper. Med., 1900, 5, p. 77.
4. Handb. d. path. Microorganismen, 1913, 5, p. 211.
5. Jour. of Med. Research, 1910, 23, p. 137.

To the naked eye, the appearance of sporothrix cultures is at times striking. Under suitable conditions, which will be more fully discussed later, the growth of the organism assumes a brown or brownish-black color. As a rule, the upper part of the culture is more intensely colored than the lower; often, indeed, the lower part of the growth just above the water of condensation is quite white while the upper part is quite black. Again different parts of the surface growth may vary markedly in color, certain areas, or colonies, being gray or white, surrounded by a black field, or the reverse may be true. Other curious and irregular distributions of pigmented and non-pigmented growths in the same culture tube may occur. They are inconstant in many respects, even under apparently identical conditions, and therefore difficult to explain.

Under apparently the same conditions, different strains do not behave alike, there being considerable variation in the time of the appearance of the pigment, its intensity, shade, and distribution. The pigment may first appear along the margin of the growth on slant media, as a blackish-brown line, and as a rule the coloration is deeper along the margins than toward the center. But there are frequent exceptions to this. The pigment may first be seen along a ridge of growth or at the crown of a slight elevation, or hillock, of growth, both of which so often occur on the slants. Later, the color may spread and involve the entire surface, or it may become permanently limited to certain regions.

The growth on all media at first is white. The time of appearance of the pigment varies greatly. In some strains it begins to be visible in about a week or ten days; in others it may not appear for three or more weeks. At times it forms rapidly, the growth in one night becoming decidedly darkened; or the pigmentation may be a very gradual process, the media becoming slowly darker over a period of weeks.

If cultures of sporothrix are examined under the microscope, it readily may be seen that the spores are the chief seat of pigment formation. In unstained preparations placed under a cover glass, the pigmented spores can be differentiated from the non-pigmented ones. In cultures showing no pigment grossly all the spores are quite colorless. In the pigmented cultures, both non-pigmented and pigmented spores are always seen. The spores have a brownish tint, which varies considerably in intensity. Tho the growth in the tube may appear black to the naked eye, the spores under the microscope

are never more than moderately brown in color. Some spores apparently never acquire any color. Some of these are probably young, for young cultures, even tho profuse, are always colorless.

Figure 1 is intended to present the color of spores, as they appear under the microscope, from a culture which was very dark brown, or nearly black, to the naked eye. To obtain the proper effect the microscopic illumination should be carefully regulated by diminishing the intensity of the light to the proper degree. It will be observed that the pigment is absolutely homogeneous, there being no suggestion of granular structure even with the highest magnification. It is also uniformly distributed in the spore. I have not observed more intensive coloring of one side, or part, of a spore than another. The mycelium when seen in mass under the microscope may appear light brown, undoubtedly as the result of pigment; but the pigment is difficult to observe under high power and in individual filaments. In the filaments are seen at times small granular masses, highly refractile, which with proper illumination appear faintly brown in color. These also probably contribute to the coloring of the growth. At times one may obtain cultures, or parts of cultures, made up of masses of mycelia and quite free from spores. Such masses to the naked eye are usually whitish or very pale yellowish-brown, and never, so far as I have observed, deep-brown or black. The latter are always rich in spores. The evidence, then, points to the presence of a small amount of pigment in the mycelial growth, but to by far the greater portion in the spores. It may be pointed out further that pigment is never seen outside the spores or the mycelial filaments in either granular or diffuse form.

In addition to the brownish-black pigment, there is also another pigment, ordinarily less conspicuous, which is yellow or yellow-brown in color. This seems to be distinct from the brown-black pigment, but the two often exist together. The yellowish pigment is commonly seen distinctly concentrated in colonies of the organism. At times, indeed, colonies growing on the surface may be conspicuously yellow, especially when viewed from below. On agar cultures, especially sugar agar, the media as a rule come to have in the course of one to three weeks the color of honey. A layer near the surface at first becomes more intensely colored, tho the coloration is never very deep; later, usually the media assume this yellow-brown, or honey-colored appearance, throughout. This pigment is evidently diffusible in the media.

It is to be noted that this coloring of the media is different from the slight alteration due to the growth of the fungus into the media. The latter may occur to a considerable degree under certain conditions and may alter somewhat the color of the involved medium.

The discoloration of the media noted by some writers is, I take it, due to the diffusion of this yellowish or yellow-brown pigment into it, and not to the blackish or black-brown pigment of the spores, which, as we shall presently see, is quite indiffusible.

An attempt was made to determine some of the properties of the pigment. For this purpose, deeply pigmented growths several weeks old were used. The mass removed from the surface of glucose agar plates was black to the eye, and portions quite free from the media were subjected to various fluids in order to determine the solubility of the pigment. It was found to be highly insoluble. Water, alcohol 95 percent, and absolute, cold or hot, ether, xylol, chloroform, acid alcohol, weak alkalies, and weak acids appeared to have no effect. Strong HNO_3 and H_2SO_4 quickly dissolved the entire organism and destroyed the pigment. Therefore it appears that the pigment is insoluble in aqueous solutions, and indiffusible in fat solvents, weak acids, and weak alkalies.

The pigmented spores in their staining reactions appear to behave exactly as do the non-pigmented ones. The pigment does not give the iron reaction.

The character of the media with respect to chromogenesis is important. On fluid media, if the growth is permitted to remain for some time on the surface, a thick mat forms in which a brownish or blackish pigment may appear. Ordinarily, however, the growth in the fluid sinks to the bottom in floccular form and when in this position remains uncolored. This is probably due, as will be shown later, to the diminished supply of oxygen beneath the surface of the fluid. On account of the peculiar way in which the growth occurs in fluid media, the latter are not satisfactory for the study of pigment production.

Solid media furnish more suitable conditions; for here, at least on certain kinds of solid media, pigment may be observed constantly. On ordinary plain agar, as it is usually made with meat or meat extract, some pigment is often produced, but as a rule not in abundance. On sugar media, pigment is more abundant. The statement has been made that if impure sugars are used — for example, the common, dark-colored, impure glucose — the pigment is more intense and appears earlier in the cultures. I am not sure that this is so. The growth is as a rule more abundant on such media, and this profuse growth may be the cause of the more abundant pigment.

On account of the complexity of ordinary media, it was difficult or impossible satisfactorily to analyze results obtained thereon; consequently, the growth of the sporotricha was tested on a series of synthetic media made up in a variety of ways. On media containing 2 percent pure agar plus 0.5 sodium chlorid, there is always some growth but it is very slight. However, even here, slight but distinct brown or black pigment may be produced at times. The growth appears after a few days as a white layer on the surface but soon stops altogether. There is never formed the thick, heavy, corrugated growth seen on ordinary plain or sugar agar. On media containing agar 1.5 percent, sodium chlorid 0.5 percent, and pure glucose 2.0 percent, the growth is also very scant, being little or no more than in the absence of glucose. The pigment formation is also slight, but at times it is distinct. The same holds true for this media

if impure glucose is substituted for the pure glucose. In the following synthetic medium, agar 1.5 percent, asparagin 2 percent, $MgSO_4$ 1 percent, K_2HPO 1 percent, sporotricha grow scantily, but on the whole perhaps slightly better than in the simpler media. Pigment may occasionally be formed in small amounts. If glucose pure or impure is added, the growth may be slightly accelerated, but the difference is not great; indeed, often inappreciable. Likewise, pigment production is not appreciably increased, tho it is often present in small but definite amounts. It is when the proteid constituents are added that the difference becomes more marked, the growth being then much more profuse and the pigment production more intense. After many observations, I have not been able to convince myself that more or intenser pigment is formed in the presence of impure glucose than of pure glucose.

Maltose is a most excellent medium for growth and for pigment production. Saccharose, lactose, inulin, and raffinose each furnish a fairly good medium for its growth, but apparently not as favorable either for growth or for pigment production as maltose and glucose. Media containing blood give good growth but do not to any extent alter or modify pigment production.

Sterile sliced carrot is a most excellent medium both for growth and pigment production. According to my experience, it gives more intense and more constant pigmentation than any other medium that I have tried. The color of the growth of many strains is a shiny, deep black. The carrot tissue is not stained brown or black, even by the most intense pigment producers.

Often on potato, pigment is produced in abundance, and the potato beneath the growth may become dark in color. This is not due however to a diffusion of pigment.

On sterile animal tissues, growth is abundant and usually appears as a gray covering without definite or intense pigmentation. The organism grows into the tissue slowly, not in the form of filaments, but in the form of spindles, which are found there in large numbers. These are never pigmented, tho the spores forming on the surface may show some color under the microscope.

All strains of sporotricha thus far reported have been aerobes. I have tested twelve different organisms, including strains from man and horse, and none gave any appreciable growth in anaerobic tubes. The spores remain alive for a considerable time under these conditions, as can be shown by admitting air into the tubes later. In this manner, it was shown that growth occurred after an anaerobic exposure of six weeks. At the end of three months no growth appeared. If a small amount of oxygen only is admitted to a culture tube, growth will occur but it is retarded. While growth was being tested under these conditions, it was noted that even with the best pigment-producing strains no pigment was formed, but instead the media were covered as a rule with a diffuse, pure white, often snowy growth, usually without corrugations or ridges. This was true regardless of the kind of media used. After a number of methods had been tried, it was found that simply plugging a tube immediately after inoculation with a solid rubber cork diminished the amount of available oxygen sufficiently. As a rule, in such preparations, no pigment whatever appears. Occa-

sionally a small amount may be seen underneath the diffuse white growth, but it does not appear on the surface. This is rare, however, and when present it is probably due to a slight excess of oxygen. When air is admitted, pigment will begin to form in the course of several days.

In stab cultures, no pigment ever appears in the growth in the depths of the media, while on the surface pigment may be abundant. The spores which form beneath the surface in such cultures, when examined for pigment with the microscope, are likewise always found to be colorless. This absence of pigment formation in the depths of the medium is probably due to an insufficient supply of oxygen at that place.

Experiments were designed to test the effect of light on the growth and pigmentation of cultures of sporothrix. Tubes inoculated and placed at once in an absolutely dark chamber continued to grow and to produce pigment in the ordinary manner. No essential differences were noted between the tubes kept in the dark and the control tubes placed in diffuse light. In sun light, too, no appreciable alterations were noted, tho the growth in certain tubes was somewhat retarded. The pigment soon appeared, and in intensity closely corresponded with the control tubes placed in diffuse light and total darkness.

In certain strains of sporotricha, there has been noted a striking distribution of pigment. The pigment occurs in well-defined regions, the remainder of the growth remaining white indefinitely, thus being produced what I have come to call spotted cultures. If the inoculation is scant so that individual colonies appear on the slant or plate, certain colonies are deeply pigmented and others are white. I do not know what causes this phenomenon. I have not noted it in connection with all strains; it was especially noticeable in a strain which I isolated from a human case reported by Dr. Hyde and myself in 1910. At times, in other cultures, the pigmented portions are not so clear-cut and well-defined, the white and the pigmented areas fusing more gradually.

If one carefully makes subcultures on maltose agar or carrot from the white and from the pigmented regions, or colonies, one often obtains white and pigmented cultures, respectively, which reproduce true (Fig. 2). Mixed cultures not infrequently result, but by carefully selecting the material one will obtain what appears to be a pure white and a pure black, or pigmented, strain. I have several such strains and have carried them through eleven generations, each generation growing

for at least six weeks before being again transferred. It is necessary to observe the cultures about this length of time in order to know whether or not pigment will be produced by the culture. Thus far they have bred true, the white producing white and the black producing black. Also many ordinary subcultures, made on media suitable for pigment production, likewise have thus far bred true. It is impossible of course to say what such cultures may do in the future and they should be kept under observations for years to determine this. At the present time I can state only that I have white strains which under the most favorable conditions for pigment production that we know, have failed to produce it during a period of observations of about sixteen months. The black strains, coming from exactly the same original human strain as the white, produce intensely black pigment. I have obtained from some, but not all, the black cultures some white ones. This has probably been due to a mixture of white and black in the pigmented colonies, which might easily occur.

The white and black strains differ in certain respects other than pigment production. The surface of the black cultures is more wrinkled and corrugated, whereas the surface of the white is more velvety, or appears powdered. Small, fine spicules are often present on both pigmented and non-pigmented strains. Spores are less abundant on the white than on the black cultures. The spores of the white cultures, when examined under the microscope, are always seen to be entirely free from color. The mycelia of the two are, however, not different.

When inoculated into animals (white rats), the black and the white strains appear to be equally pathogenic. Cultures from the lesions thus produced in these animals give rise to pure white and black varieties, identical with those injected.

Dr. Moon, by means of the Barber technic, isolated for me single spores from the black and from the white varieties. From these there developed white and black subcultures, respectively. Such strains have been carried through ten generations and have bred true.

As to the significance of this phenomenon, I think we should be conservative. It seems to indicate that at least certain strains of these organisms are readily subject to variations; possibly, we might apply the term mutation to such changes. I think however that we should be cautious about multiplying new varieties of organisms of this type based on slight variations in cultural or morphologic properties. On the other hand, it should be pointed out that if these white strains

continue to breed true and retain permanently their present properties, they will be different from all the strains that have been cultivated directly from the lesions in man or in animals. As has been pointed out, all these, under proper conditions, are pigment producers.

SUMMARY

All cultures of pathogenic sporotricha that have been isolated and carefully studied are pigmented when grown on suitable media.

The color varies, being black or shiny black, dark brown, chocolate brown, slight brown.

The time of the appearance of pigment on cultures varies considerably; it is usually from one to several weeks after inoculation.

Media most suitable for pigmentation are carrot (sliced and sterilized), potato, 3 percent maltose agar, and 3 percent glucose agar. Other sugars are not so useful for this purpose.

Cultures remain white in an insufficient supply of oxygen. Abundant oxygen seems to be necessary for chromogenesis.

Sunlight, diffuse light, and absolute darkness have no appreciable effect on chromogenesis.

The pigment is insoluble in water, acids, alkalies, and in fat solvents (ether, alcohol, chloroform, benzol, xylol).

The slight darkening of the media under the growth of sporothrix is probably due to a second pigment of a yellowish color produced by this organism.

The color lies almost entirely in the spores on the surface of cultures. Mycelium and spores in the depths of the medium are colorless, the latter probably because of lack of oxygen.

In certain cultures, pigmented and white regions, or colonies, appear. From such growths pure pigmented and pure white strains have been obtained.

When passed through animals (rats), these white and black strains remain pure.

Single spores from the white and the black cultures give rise to pure white and black subcultures, respectively.

EXPLANATION OF PLATES 2 AND 3

Fig. 1.—Pigmented and colorless spores from a culture of sporothrix which was quite black to the naked eye.

Fig. 2.—A. Culture of sporothrix from man which shows well defined regions of pigmented and non-pigmented growth.

B. Subculture on maltose agar made from pigmented area of culture A.

C. Subculture on maltose agar made from white areas of culture A. B and C were grown under identical conditions.

PLATE 2

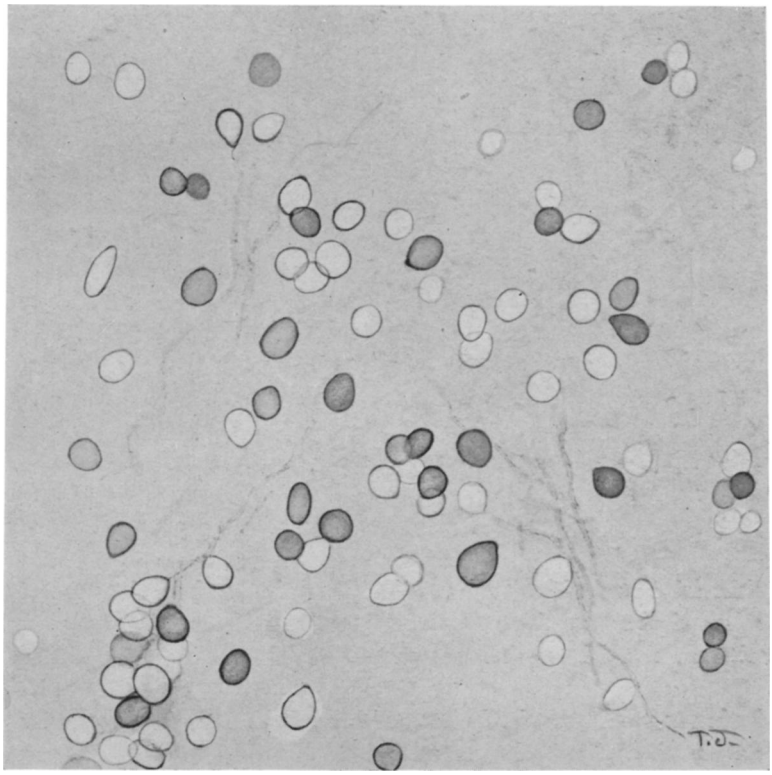

Figure 1

PLATE 3

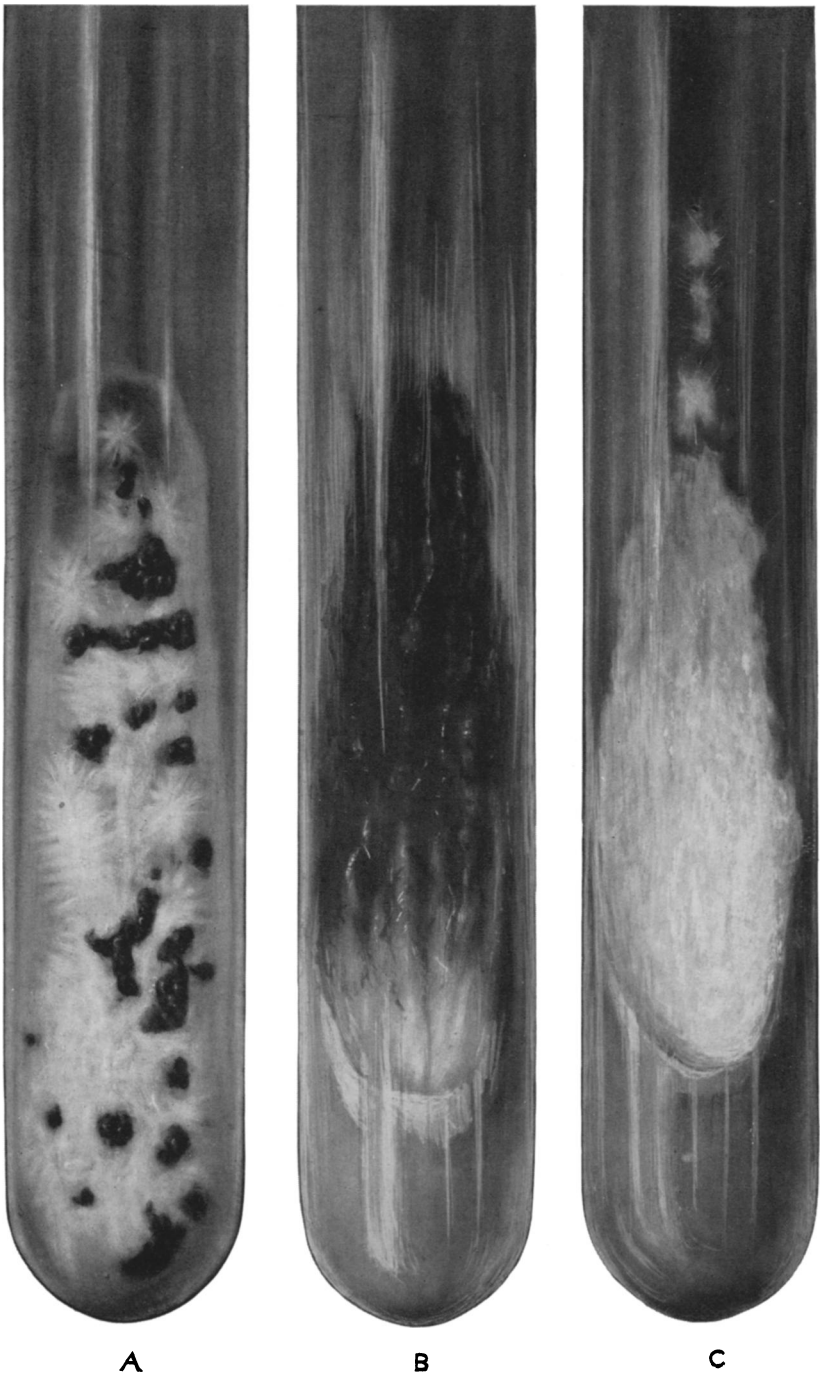

Figure 2